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Assessment of Bacterial Mutagenicity Methods for Volatile and Semi-volatile Compounds and Mixtures

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16. ABSTRACT

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ASSESSMENT OF BACTERIAL MUTAGENICITY METHODS FOR VOLATILE AND SEMIVOLATILE COMPOUNDS AND MIXTURES[a]

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Extensive effort has been given to identifying and quantitating the organic genotoxins associated with airborne particles; however, little is known about the mutagenicity of vapors and gases, especially those associated with ambient air samples. This overview of the literature and recent U.S. Environmental Protection efforts examines some of the critical aspects associated with the bacterial mutagenicity testing of gases and vapors. For example, the sensitivity of the test system depends upon the method of exposure (preincubation, gases over plates, liquid suspension, etc.); and the method of choice appears to depend upon vapor pressure and the solubility of the compound under investigation. Also, the selection of test chamber and other materials is important, since plastic petri dishes have been shown to absorb up to 85% of any haloalkane vapors within an exposure chamber. Investigations have also shown the importance of exposure rates, method of gas mixing, role of metabolic activation systems, and the occurrence of photoactivation. In ambient studies, the identification and quantitation of volatile organic mutagens is quite difficult; however, due to their abundance these efforts are important.

Introduction

Duce (1978) speculated that the amount of ambient air, vapor-phase organics (VPOs) is generally 10 to 100 times greater than the amount of ambient air, particulate organics. The worldwide emission rate for these anthropogenic organics is estimated to be approximately 6.5×10^{13} g vapor-phase organics per year. According to Hughes *et al.* (1980), about 25% of these vapor-phase organics are released within the United States each year. During recent years extensive effort has been given to identifying and quantitating the organic genotoxins associated with airborne particles (Waters *et al.*, 1978, 1980, 1983; Tice *et al.*, 1980). Although some advancement in the bioassay of vapor-phase organics has been made in the last decade, little is known about the mutagenicity of vapors and gases, especially those associated with ambient air samples (Hughes *et al.*, 1980).

This lack of knowledge is due to two primary, technical difficulties. First, appropriate bioassay exposure systems have not been adequately developed and characterized (Barber *et al.*, 1981). Most studies in this category appear to deficient in one or more areas (for example, not knowing the actual exposure dose of the vapor, using improper chamber materials, not providing for proper mixing, allowing confounding factors to exist or be generated within an experiment). Second, collection methods for ambient VPOs have either collected insufficient material for bioassay or have generated artifacts. Artifact problems have arisen for a variety of reasons including the following: (1) upon collection, the abundance of water in the atmosphere dilutes the organics of interest and/or helps to promote an artificial interaction of organics, NO_x, SO_2, etc.; (2) sorbent material may contain small amounts of bioactive material; (3) atmospheric exposure of a sorbent material (e.g., nitrous acid) may generate a bioactive material not normally present; (4) due to collection efficiencies of a sorbent and/or breakthrough volumes for particular organics, the collected material may not be representative of the air volume sampled; and (5) the concentration of organic material may contribute to the *in situ* formation of artifacts (Bursey *et al.*, 1977; Pellizzari, 1978).

[a]Although this paper has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC, it has not been subjected to policy review and therefore does not necessarily reflect the views of the Agency and no official endorsement should be inferred.

The purpose of this paper is to assess the state-of-the-art for the bacterial mutagenicity testing of organic vapors and gases. This assessment includes a brief overview of work done by other investigators and recent method development efforts of the U.S. Environmental Protection Agency, Research Triangle Park, NC.

Overview of Published Results

Most of the research done with bacterial mutagenicity assays has dealt with individual compounds and simple exposure systems. (Since most of the investigations have used strains of *Salmonella typhimurium*, all strains designations will refer to these *Salmonella* strains unless otherwise stated.) Table 1 provides a summary of the work reviewed within this paper.

Bartsch *et al.* (1975b) tested vinyl chloride (VC) using two methods. One method described as "incubation in liquid suspension" (0.083 M VC) yielded no increase in mutant numbers when strains G46 and TA1530 of *Salmonella typhimurium* were used. The second method, in which plates containing bacteria and a 9000 xg liver supernatant (S9) were exposed to VC vapors within a glass desiccator, gave a positive response with strains TA1530, TA1535, and G46. Researchers, therefore, have realized for nearly a decade that the method of the organism's exposure was important. A number of other organic compounds, including vinylidine chloride (Bartsch *et al.*, 1975a; Jones and Hathway, 1978), 2-chlorobutadiene (Bartsch *et al.*, 1975a), and dichloromethane (Jongen *et al.*, 1978) have given positive test results in similar vapor-phase systems.

When working with a series of halogenated organic compounds, Rosenkranz (1977) realized that it was impractical to test these compounds in the normal plate incorporation technique, due to the volatile nature of many of these substances. In order to test these compounds in a more qualitative manner, chemicals were deposited onto filter disks and a spot test was performed. Most of the compounds tested were mutagenic to strain TA1535 (Table 1).

In 1978, Bridges compared a variety of methods when assaying the two volatile compounds dichlorvos and epichlorhydrin. The dichlorvos was tested with the WP2 strain of *E. coli* and the epichlorhydrin was tested using strains of *S. typhimurium*. Although each of the methods gave positive results, Bridges observed that the use of glass petri dishes and continuous exposure enhanced the sensitivity for detecting mutagens. He also noted that "the distribution of mutagen between gas and aqueous phases differs for different substances."

McCoy *et al.* (1978) compared three methods for volatile compounds using allyl chloride. In the standard plate test, allyl chloride is negative. When the compound was placed on filter disks assayed in spot tests done in sealed bags, it was positive in TA1535. It was also positive using TA1535 with a preincubation protocol.

In 1978 deMeester *et al.* demonstrated an interesting phenomena for the volatile compound vinyl chloride (VC) whose activity is enhanced by the presence of S9. This work assayed VC as a vapor within a desiccator using strain TA1530. In the first experiment, when plates with bacteria but no S9 were exposed to VC, a weak response was seen. When the desiccator contained some plates with S9 and some plates without S9, an increased response compared to the first experiment was seen on *both* types of plates. In the third experiment some plates contained "only S9" while other plates contained "only bacteria." Again, when compared to the first experiment, there was an increased mutagenic response. These results indicate that volatile metabolites can influence results upon adjacent plates.

Chromyl chloride was applied to the lids of petri dishes which were then placed inside gas-tight jars (de Flora *et al.*, 1980). When the bacteria were exposed in this manner, either continuously or for 5 min, this compound gave negative results. When only the minimal base agar was exposed in this manner and the bacteria subsequently overlayed onto the exposed media, a positive response was seen. A positive response was also elicited when the compound was placed on a filter disk and a spot test was done.

In order to overcome some of the limitations of previous studies, Barber *et al.* (1981) designed and utilized a chemically inert, closed incubation system for glass petri dishes. This system allowed vapor-phase headspace sampling so that gas-liquid chromatographic analysis of vapor-phase chemical concentrations could be made. The system was evaluated using ^{14}C-labelled 1,2-dibromoethane. Seven of 10 solvents were shown to be mutagenic in this system, although only 3 of 10 are positive in the plate incorporation assay.

Using 1,2-dibromo[^{14}C]ethane, Barber *et al.* demonstrated that approximately 85% of the organic vapor could be scavenged by the plastic petri dishes and approximately 14% was associated with the agar medium. When glass petri dishes were used, 60%–80% of the total dose was found associated with the agar. The actual percentage depended upon the dose administered. Using two of the solvents they also showed that, although the kinetics of entry of solvents from the vapor phase into the aqueous phase are complex, approximately 80% of the solvent was removed from the air in 1 h. Due to these results, Barber *et al.* suggest that all compounds boiling a temperature less than 200 °C will show a diminished response in the standard plate test due to volatilization.

Few investigators have examined for mutagenicity of complex mixtures of organic vapors. Hedenstedt *et al.* (1981) used a water-cooled glass condenser to condense the vapors that resulted from the pyrolysis of various types of rubber. Each condensate was then tested in the standard plate test; and varying degrees of toxicity and mutagenicity were observed depending upon the type of rubber pyrolyzed, were seen. Penalva *et al.* (1983) ex-

Table 1. Summary of published methods used for the bacterial mutagenicity testing of volatile organic compounds and mixtures.

Author(s)[a]	Method(s)	Compound(s)	Results: Activity[b]	Results: Strain/Condition[c]
Bartsch *et al.* (1975)	A. Vapors within desiccators	Vinyl Chloride	+	TA1530/TA1535/G46 : +S9
			−	TA1538 : +S9
	B. Liquid Suspension (unsealed)		−	G46 : +S9
			−	TA1530 : +S9
Bartsch *et al.* (1975)	Vapor within desiccator	Vinylidene chloride	+	TA1530/TA100 : +S9
		2-Chlorobutadiene	+	TA100 : −/+S9
Rosenkranz (1977)	Liquid on filter disks	1,2-Dibromoethane	+	TA1535/TA1530
		1,1-Dibromoethane	+	TA1535/TA1530
		1,2-Dichloroethane	+	TA1535/TA1530
		1-Bromo-2-chloroethane	+	TA1535/TA1530
		1,3-Dibromopentane	+	TA1535/TA1530
		1,2-Dibromo-2-methylpropane	+	TA1535/TA1530
		1,1,2,2-Tetrachloroethane	+	TA1535/TA1530
		1,1,2,2-Tetrabromoethane	−	TA1535
		2-Chloroethanol	+	TA1535
		2-Bromoethanol	+	TA1535
		2-Chloroacetaldehyde	+	TA1535
		2-Bromoacetaldehyde	−	TA1535
		Acetaldehyde	−	TA1535
		2-Bromoethylacetate	+	TA1535
		(Above 14 compounds)	−	TA1538
Bridges (1978)	A. Liquid Suspension	Dichlorvos	W+	WP2 trp *E. coli*
		Epichlorhydrin	W+	G46, TA100
	B. Fluctuation	Dichlorvos	+	WP2 trp *E. coli*
	C. Agar Incorporation (sealed)	Dichlorvos	+	WP2 trp *E. coli*
			+	CM881 trp *E. coli*
		Epichlorhydrin	+	TA100
	D. Agar Incorporation (unsealed)	Dichlorvos	+	WP2 trp *E. coli*
			+	CM881 trp *E. coli*
	E. Vapor (sealed)	Dichlorvos	W+	WP2 trp *E. coli*
		Epichlorohydrin	+	TA1535/TA100
	F. Vapor (sealed, glass perti dishes)	Dichlorvos	+	CM881 trp *E. coli*
		Epichlorohydrin	+	TA100
Jones and Hathway (1978)	Vapor in gas-tight culture vessels	Vinylidene chloride	+	TA1535 : +S9
Jongen *et al.* (1978)	Vapor in gas-tight perspex boxes	Dichloromethane	+	TA98/TA100 : −/+S9
McCoy *et al.* (1978)	A. Standard plate test	Allyl-chloride	−	TA1535 : −/+S9
	B. Compound-impregnated disk, spot test in individual BioBags®		+	TA1535/TA100 : −/+S9
			−	TA1538 : −/+S9
	C. Pre-incubation		+	TA1535 : −/+S9
deMeester *et al.* (1978)	Vapors within desiccator under following conditions:	Vinyl chloride		
	A. Bacteria only plates		+	TA1530 : −S9
	B. Bacteria only and +S9/ Bacteria plates		+	TA1530 : −S9/+S9 (−S9 plate gave increased response)
	C. Bacteria only and +S9 only plates		+	TA1530 : (+S9) (−S9 plates also gave an increased response)
De Flora *et al.* (1980)	A. Liquid in standard plate test	Chromyl chloride	+	TA100 : −/+S9
	B. Liquid applied to plate lid in gas-tight jar. Continuous exposure		− (Toxic)	TA100 : −/+S9

Table 1. (Continued)

Author(s)[a]	Method(s)	Compound(s)	Results: Activity[b]	Results: Strain/Condition[c]
	C. Liquid applied to lids in gas-tight jar. 5 min exposure; lids replaced.		− (Toxic)	TA100 : +/+S9
	D. Bacteria overlayed onto plate after minimal base agar exposed to vapors.		+	TA100 : −/+S9
	E. Vapor-exposed disk used in spot test.		+	TA100 : −/+S9
Barber *et al.* (1981)	Vapor in closed, inert system	1,2-Dibromoethane	+	TA1535/TA98/TA100 : −/+S9
		Bromoethane	+	TA1535/TA100 : −/+S9
			−	TA98 : −/+S9
		Iodoethane	+	TA1535/TA100 : −/+S9
			−	TA98 : −/+S9
		1-Bromopropane	+	TA1535/TA100 : −/+S9
			−	TA98 : −/+S9
		1-Bromo-2-chloroethane	+	TA1535/TA100 : −/+S9
			−	TA98 : −/+S9
		1-Bromobutane	+	TA1535/TA100 : −/+S9
			−	TA98 : −/+S9
		1,2-Dichloroethane	+	TA1535/TA100 : −/+S9
		1,1,2-Trichloroethane	−	TA1535/TA100/TA98 : −/+S9
		1-Chlorobutane	−	TA1535/TA100/TA98 : −/+S9
		Carbon tetrachloride	−	TA1535/TA100/TA98 : −/+S9
Hedenstedt *et al.* (1981)	Vapors collected with water-cooled all glass condenser. Standard plate incorporation test.	Vapors of:		
		1. Styrene-butadiene rubber	+	TA100 : −/+S9
		2. Butadiene-acrylonitrile rubber	−	TA98/TA100 : −/+S9
		3. Chloroprene rubber	+	TA100/TA1535 : −S9
			−	TA100/TA1535 : +S9
			−	TA98/TA1538 : −S9
			+	TA98/TA1530 : +S9
		4. Ethylene propylene rubber: sulfur-cured	+	TA100 : −/+S9
			+	TA98 : +S9
			−	TA98 : −S9
		5. Ethylene propylene rubber: peroxide-cured	+	TA98/TA100 : −/+S9
Penalva *et al.* (1983)	Vapors collected in wash bottles with DMSO	Road-coating tar		
	A. DMSO vacuum evaporated		W+	TA98 : −/+S9
			+	TA100 : −S9
			?	TA100 : +S9
	B. Extraction with *n*-hexane, solvent exchange		+	TA98 : −/+S9
Ong *et al.* (1984)	*In situ* liquid impinger system	Methylmethane sulfonate	+	TA100W : −S9
		Ethylmethane sulfonate	+	TA100W : −S9
		Ethylene dibromide	+	TA100W : −S9
		Ethylene oxide	+	TA100W : −S9
		2,4,7-trinitro-9-fluorenone on silica particles	+	TA98W : −S9
		2-aminoanthracene	+	TA98W : +S9
		Dimethylnitrosamine	+	TA100W : +S9
		Cigarette smoke	+	TA98W : +S9
		Diesel Engine Emissions	+	TA98W : −S9

[a]Listing of papers is chronological; see reference list for complete reference.
[b]Activity: + = positive; − = negative; W+ = weak positive; ? = questionable.
[c]Strain/Condition: All strains are *Salmonella typhimurium* unless otherwise noted. + TA98/TA100 : −/+S9 means that the compound is positive in strains TA98 and TA100 with both −S9 and +S9 conditions. Some authors did not report the S9 conditions and this section is left blank.

amined solvent collected emissions from heated road-coating tar and found this condensate mutagenic.

In order to assess the mutagenicity of workplace air, Ong *et al.* (1984) developed an *in situ* monitoring system that uses bacteria. In this system, air is bubbled through a bacterial broth culture placed inside a 100-mL pear-shaped glass flask. Since these cultures can be easily contaminated, the broth is supplemented with 100 μg 8-azaguanine/mL, 10 μg cycloheximide/mL, 100 μg streptomycin/mL, and 25 μg ampicillin/mL. The bacterial strains used with this system (TA100W and TA98W) are resistant to these antibiotics. Preliminary work with this system (Table 1) shows that this system can detect complex mixtures as well as individual compounds that are mutagenic.

Review of Recent U.S. Environmental Protection Agency, Research Triangle Park, NC, Experience

With the exceptions noted for the three methods, the bacterial bioassay was performed according to the method of Ames *et al.* (1975). *Salmonella typhimurium* strains TA100 and TA98 were kindly provided by Professor B. Ames, University of California, Berkeley, CA. The 9000 g liver supernatant (S9) was prepared from 1254 Aroclor induced CD-1 rats (150–220 grams) according to the method of Ames *et al.* (1975). Mutagenicity assays were performed in one of three days. Each of these three methods and results will be described more fully in separate publications that are in preparation.

Method A

This method was carried out by incubation in liquid suspension within a closed system consisting of a one-dram glass vial with a Teflon-coated cap. The preincubation was performed at 37 °C in a temperature-controlled exposure chamber (Isotemp, Fisher Scientific). After incubation, the bacteria was plated upon selective media to determine the number of revertant colonies. A complete description is given elsewhere (Hughes *et al.*, in preparation). The protocol was modified to test the effect upon mutagenicity of the following parameters: (1) log- and stationary-phase cells, (2) headspace volume, (3) type of shaking, (4) choice of solvents, and (5) time of preincubation (10 to 60 min).

Method B

Method B consists of four biochambers designed to test not only the substance of interest but also the products of that substance after atmospheric interaction and transformation. The reaction chamber, originally referred to as the Mobile Aerosol Reaction Chamber, is a continuously stirred tank reactor (CSTR) described elsewhere (Parks, 1982). In essence the chamber is a large cylindrical Teflon® bag (2 m diam × 8 m length) held between two aluminum plates that are Teflon® coated. This flow-through system provides a steady-state stream of reaction products to two of the bioassay chambers.

Four bioassay chambers (Fig. 1) were used for different types of exposures. Chamber A was used to expose bacteria to air from the clean air source. Chamber B was used to expose plates to the reactants before entry into the reaction chamber. The unfiltered products of the reaction chamber were used to expose bioassay plates in Chamber C. The exposure to filtered products of the reaction vessel in Chamber D was achieved by using a Pallflex Teflon-coated glass-fiber filter. Up to 50 plates containing the various bacterial strains, either with or without S9, were placed in each chamber and exposed for 20 h. By adjusting the residence time (either 2.7 or 6.7) of the organic reactants within the CSTR, one can examine different steady-state conditions.

Method C

Mutagenicity assays for selected compounds were performed by exposing test plates of bacteria to the selected agent in Billups-Rothenberg chambers (Billups-Rothenberg, Flow Labs, McLean, VA) for 24 h at 37 °C in the dark. The chambers were then evacuated and purged with clean air. The plates were then incubated at 37 °C in the dark for an additional 24 to 48 h. Twelve plates were placed inside each chamber, and separate chambers were used for each dose tested. A more complete description of this system is in preparation (Claxton and Perry, in preparation).

Results of Recent U.S. Environmental Protection Agency Studies

Since the purpose of this publication is to compare the various types of approaches to the testing of volatile agents and since some of the results are in preparation (Claxton and Perry; Hughes and Claxton), only representative results are given.

Butylene oxide was tested using the liquid suspension assay in closed vials protocol (Method A). This compound was tested using a variety of conditions (Fig. 2). Three types of comparisons made were as follows: (1) half-full vs. full vials, (2) type of exogenous metabolic activation (rat vs. hamster vs. none), and (3) the type of shaking during incubation. For butylene oxide, the system was most responsive when full vials were used with no shaking. Exogenous metabolic activation had little effect upon this compound. Preliminary results indicate that the most appropriate conditions depend upon the volatility and chemical nature of the compound and that one cannot easily generalize as to which conditions are most appropriate with this method.

The use of the CSTR (Method B) demonstrates that the atmospheric transformation products of a simple nonmutagenic hydrocarbon, propylene, are mutagenic. Table 2 shows that the reactants, clean air chamber, and laboratory spontaneous control plates gave similar

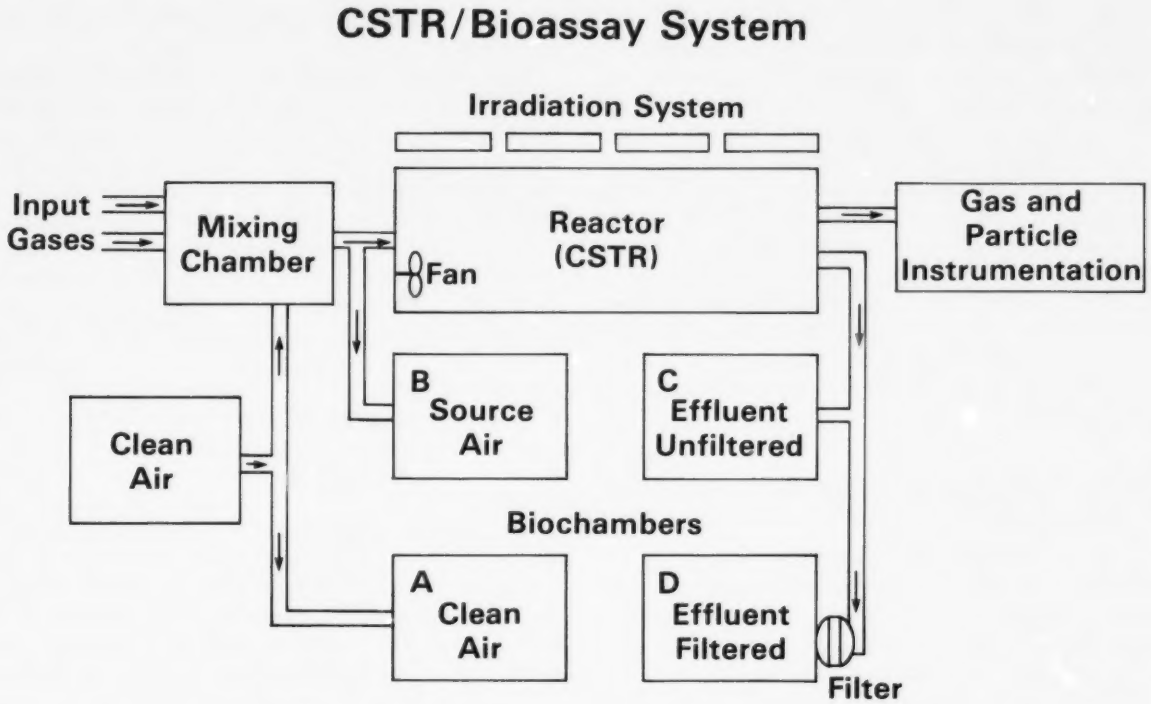

Fig. 1. The continuously stirred tank reactor system (CSTR) configuration with biochambers.

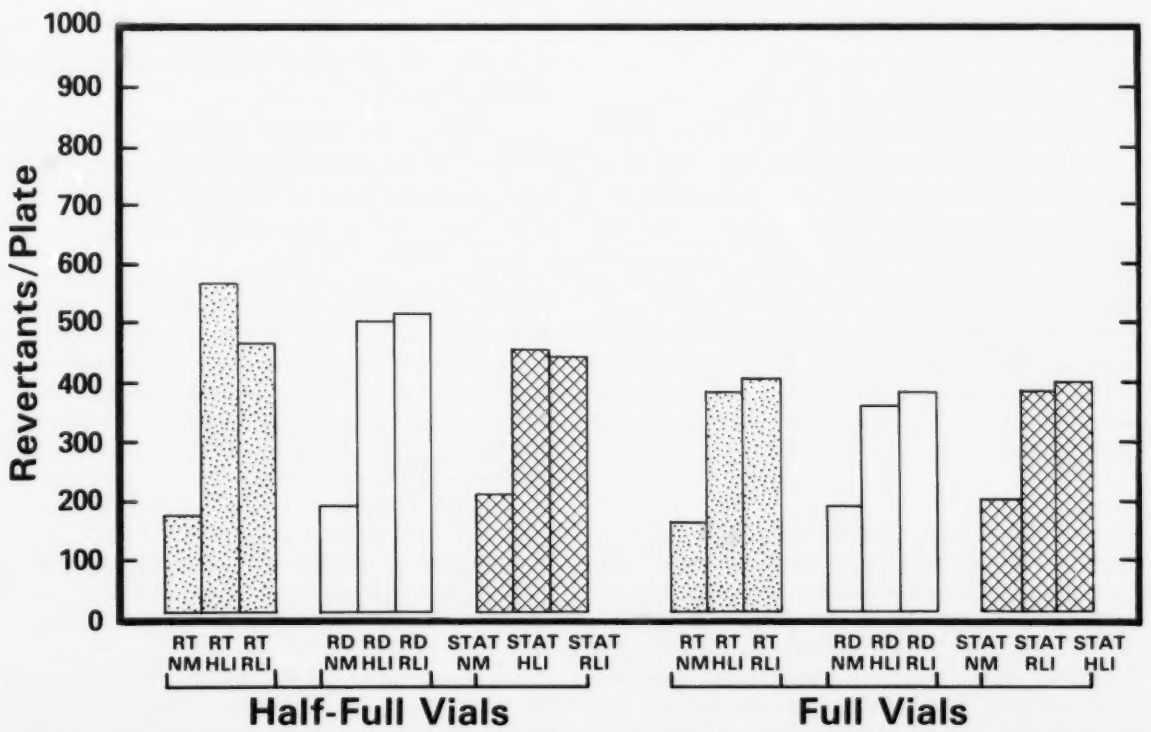

Fig. 2. The mutagenicity of butylene oxide tested using different preincubation conditions. RT: rocker table used for mixing; RD: rotating drum used for mixing; STAT: vials remained stationary; NM: exogenous activation system not used; HLI: induced hamster liver activation system; and RLI: induced rat liver used for activation.

Table 2. The *Salmonella typhimurium* TA100 mutagenicity results for the continuously stirred tank reactor (CSTR) experiments with propene

Date	τ[a]	Mean Number of Revertant Colonies per Plate per Exposure[b]				
		Spontaneous	Clean Air A	Reactants B	Unfiltered C	Filtered D
24 May	6.7	150	147	142	551	581
8 June	6.7	122	171	174	485	572
Mean	6.7	136	159	158	518	577
1 June	2.7	78	157[c]	139	531	541
14 June	2.7	130	214[c]	169	622	625
Mean	2.7	104	186[c]	154	577	583

[a]Refers to mean residence time in hours.
[b]Mean of 10 plates.
[c]This increase over spontaneous levels has recently been shown to be due to UV exposoure (< 1 min) during plate transfer to the CSTR chamber. Ongoing and future efforts will prevent this type of exposure.

values. The mean counts for plates exposed to reactant effluents from the CSTR are, however, approximately fourfold greater than the spontaneous controls. The results of the two residence times are similar and were reproducible.

The major products of the propene/NO_x/SO_2 experiments were individually assayed using the Billups-Rothenberg chambers (Method C). Table 3 presents a summary of these results. Each of these products was mutagenic with the exception of acetaldehyde. Future efforts will explore the contribution of each of these products (and others) to the mutagenicity of the total mixture.

Discussion

Although the *Salmonella* bioassay has been used extensively for the mutagenicity testing of organics, relatively few volatile and semivolatile compounds have been tested. As seen from Table 1, most of the testing was completed in an unsophisticated manner in order to supply qualitative results. Several of these studies, however, compared methods and identified some of the key issues associated with the testing of volatile compounds.

Table 3. *Salmonella* mutagenicity of identified CSTR products from propene/NO_x/SO_2 experiments.[a]

Compound	TA100		TA98	
	−MA	+MA	−MA	+MA
Acetaldehyde	Neg	Neg	Neg	Neg
Acrolein	Toxic	Toxic	+	+
o-Cresol	Neg	Neg	+	+
Glyoxal	+	+	Neg	Neg
Methyl glyoxal	+	+	W+	Neg
PAN in heptane	Neg	Neg	Neg	Neg
PAN in vapor phase	?+	?+	?+	?+

[a]Results are recorded as: Neg = ngeative; + = positive; W+ = weak positive; ?+ = questionable positive. The −MA and +MA columns refer to without and with exogenous metabolic activation, respectively. Quantitative results are to be presented elsewhere (Claxton and Perry, in preparation).

First, the sensitivity of the system depends upon the method of exposure. For example, vinyl chloride is positive as a vapor within a desiccator but is negative in a liquid suspension assay (Bartsch *et al.*, 1975). Allyl chloride was found negative in the standard plate but was positive in spot tests using chemical-impregnated filter disks and pre-incubation assays (McCoy *et al.*, 1978). Somewhat in contrast, DeFlora *et al.* (1980) demonstrated that chromyl chloride tested as a vapor was negative; however, chromyl chloride at similar concentrations was positive in the standard plate test. The method of choice appears to depend upon vapor pressure and solubility of the compound under evaluation.

The second key issue involves the use of proper materials. Barber *et al.* (1981) clearly showed that volatile organics are absorbed by the various materials common to bacterial mutagenicity testing. Most disturbing was the demonstration that plastic petri dishes could absorb up to 85% of an haloalkane vapor within an exposure chamber. Obviously, exposure chambers made of various types of plastic would absorb organic vapors and would have varying absorption rates. When various solvents are utilized with previously exposed chambers, many organic compounds would also desorb. This could cause confusing or erroneous results in subsequent experiments.

There are a number of issues that the reviewed research did not adequately answer. Some of these issues (e.g., total time of exposure, mixing of gases or vapors, role of metabolic activation, photoactivation, etc.) are a part of our ongoing research. The results to date show that these too are important issues. For example, Fig. 2 shows that the most responsive preincubation system for butylene oxide occurs when full vials containing stationary-phase bacteria and an induced rat liver activation system were used. Unfortunately, these parameters change depending upon the nature of the substance

being tested. The complexity associated with the mutagenicity testing of ambient vapors is seen from the results of the CSTR tests with propene. The photochemical reaction products of propene, (a nonmutagenic, low molecular weight hydrocarbon) gave a reproducible mutagenic response. Although some of the mutagenic products have been identified, most of the mutagenic response cannot be accounted for by the identified products.

Apparently, the identification and quantitation of volatile, ambient air, organic mutagens will be quite difficult; however, due to their abundance, they are of great importance.

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